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CRYSTALLINE LACTOPEROXIDASE

I. ISOLATION BY DISPLACEMENT CHROMATOGRAPHY

II. PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES

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The biological importance of the heme proteins and the advantages inherent in displacement chromatography as a purification technique led to a reinvestigation of the pigmented, iron-containing fraction of milk whey. This study culminated in the isolation of a crystalline lactoperoxidase and the purification of the red iron-containing protein associated with the peroxidase.

The application of chromatography to the problems of protein chemistry has not met the success attained with more simple and well defined organic structures. The subject has been reviewed in a number of recent publications (1–4). In general, attempts to fractionate complex mixtures of proteins with a single adsorbent by variation of the ionic environment lead to limited and partly successful separations. More decisive results are obtained by imposing such conditions of ionic environment on selective adsorbents that the relative adsorptive affinities of the various components differ enough to adsorb almost specifically the protein component of interest. With the present status of chromatography, this necessitates an empirical survey of adsorbents for the particular problem.

The myeloperoxidase of Agner (5) and the lactoperoxidase of Theorell and Åkeson (6) are the only animal peroxidases isolated in relatively pure form. The latter has been reported in the crystalline state (7). All details of the method of production, however, have not been worked out definitely (6). Preparation in the pure state was successful in the spring and early summer, but not in the late autumn (7). With the following chromatographic procedures, separations of proteins were accomplished that were attained previously only by repetitive electrophoresis. It was also possible to prepare lactoperoxidase from autumn and winter milk.

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I. Isolation by Displacement Chromatography

Enzyme Assay—The peroxidase concentration of the milk fractions was determined with a modification of the purpurogallin test[1] of Sumner and Gjessing (8). The activity was arbitrarily defined as the mg. of purpurogallin formed in 20 seconds by 1 mg. of protein. Within well defined limits, the purpurogallin formed was proportional to the enzyme concentration. Because of the nature of the reaction (9, 10), color formation fell off rapidly after 20 seconds. The first order reaction kinetics indicated by Theorell were not applicable over an experimental time period sufficient to permit the calculation of valid purpurogallin numbers (6).

Protein concentrations were evaluated by the biuret reaction (11) or by the optical density at 280 mμ; casein, β-lactoglobulin, and lactoperoxidase were used as standards for whole milk, whey, and purified fractions, respectively.

Purer preparations, such as the column eluates, were analyzed spectrophotometrically at wave-lengths of 280, 310, and 412 mμ. A linear correlation was observed between the enzymatic purpurogallin activity and the ratio of the absorbancy at 412:280 $\times$ 412:310. The 412:310 absorbancy ratio serves as a correction factor for the presence of a red component with lower peroxidative activity.

Concentration by Salt Fractionation—The commercial skim milk used for isolation of peroxidase was processed most conveniently in 50 liter batches. Casein was removed by coagulation with rennet. Isoelectric precipitation at pH 4.7 or salt precipitation at 1.5 M ammonium sulfate, pH 6.0, was also feasible, but resulted in lower yields of enzyme. Fractionations could be carried out rapidly at room temperature. Prolonged filtrations, however, were made at 3°.

Step 1—The whey protein was precipitated by the addition of solid ammonium sulfate to a final concentration of 2.8 M, pH 6.0. After slow stirring for 1 hour, the suspended precipitate was filtered overnight at 3°.

Step 2—The precipitate was redissolved in water to a protein concentration of 3 per cent. Sodium tetraborate was added to 0.1 M and ammonium sulfate to 1.5 M. A gray, slimy, slow filtering precipitate formed that contained about 90 per cent of the alkaline phosphatase of the milk whey.[2]

[1] The reaction mixture consisted of 2 ml. of 5 per cent pyrogallol solution, freshly prepared, 1 ml. of 0.5 per cent hydrogen peroxide, 2 ml. of 0.5 M phosphate buffer, pH 6.0, 1 ml. of 1 per cent crystalline bovine serum albumin, and 14 ml. of water at 20°. The enzyme was diluted when necessary with 0.05 per cent albumin solution to such a concentration that the purpurogallin formed ranged from 0.5 to 2.5 mg. After a reaction time of 20 seconds, 1 ml. of 2 N sulfuric acid was added, and the dye was extracted with ether and measured as indicated by Sumner and Gjessing (8).

[2] Personal communication by Dr. C. A. Zittle of this laboratory.

Step 3—Increasing the salt concentration of the 1.5 M filtrate to 1.9 M produced a bright orange precipitate that contained xanthine oxidase and some peroxidase. The precipitate was filtered at 3°.

Step 4—All the peroxidase in the 1.9 M filtrate was precipitated at 2.5 M ammonium sulfate.

Step 5—The precipitate of Step 4 was redissolved in 0.1 M borax solution to a protein concentration of 2 per cent and reprecipitated (*a*) at 1.9 M and (*b*) at 2.3 M ammonium sulfate. After standing for 1 hour at room temperature, the *b* fraction formed a brown-green, sticky cake that floated on the surface of the salt solution. Filtration was readily accomplished through glass wool. Excess ammonium sulfate could be removed from this precipitate by kneading it into a ball.

Step 6—The yield of peroxidase could be increased by reworking the 1.9 M salt precipitate. To a 2 per cent protein solution, solid ammonium sulfate was added until the solution reached 2.0 M, and the pH was then adjusted to 4.8 by addition of 1 N sulfuric acid. Xanthine oxidase was concentrated in the precipitate; the remaining peroxidase in the filtrate was precipitated at 2.8 M ammonium sulfate. Step 6 is optional and was used only with certain lots of winter milk when an appreciable amount of the peroxidase activity was found in the 1.9 M salt fraction.

The electrophoretic composition of the *b* fraction of Step 5 is indicated in Fig. 1, *A*. The specific pyrogallol activity ranged from 1 to 4, depending on the milk and the manner of fractionation. Because of the effectiveness of subsequent chromatographic separations, it was found advisable to have as much peroxidase as possible in this fraction, even at the expense of higher purity. Preparations with specific pyrogallol activity of 1 or higher were ready for chromatography.

Chromatography—Of the many adsorbents investigated, chromatographic separation of the peroxidase from the crude concentrates was accomplished most effectively with tricalcium phosphate. Adsorption and elution of the peroxidase on the columns of adsorbent were determined primarily by the ionic strength and ion species of the solvent. The pH of the solution had little or no effect other than on the stability of the enzyme itself. The ionic environment of the peroxidase necessary for chromatography was defined readily by the effective protein separations and the apparent stabilizing action produced with phosphate solutions of the enzyme. At low salt concentrations (0.01 M), the columns of calcium phosphate were rapidly saturated with protein with little or no apparent separation of the protein components. At increased ionic strength of the solvent (0.05 to 0.1 M dipotassium hydrogen phosphate (K_2HPO_4)), the relative adsorptive affinity of the protein components became manifest in the formation of well defined green, red, and colorless bands. Although the bands were firmly bound at this ionic strength and could not be eluted with phosphate solution alone, it was apparent that with further addition of the protein solution

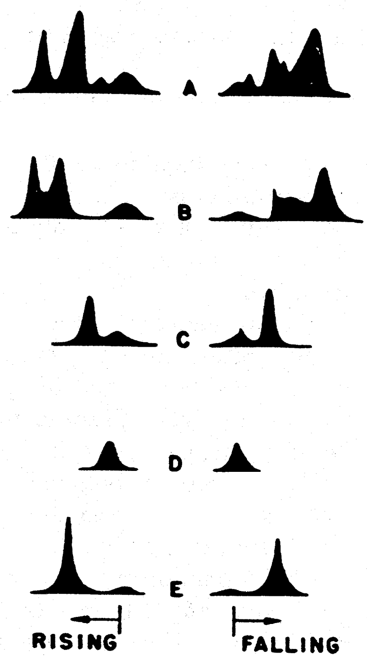

FIG. 1. Electrophoretic patterns of lactoperoxidase fractions and red protein fractions in sodium diethyl barbiturate buffer (NaCl = 0.05 M) at 0.1 ionic strength after electrophoresis at pH 8.4 for 150 minutes. *A*, b fraction of Step 5, the salt-concentrated crude lactoperoxidase before calcium phosphate chromatography; mobilities of the components on the falling side = 0.58, 2.1, 2.9, 4.0, and 4.9 u, respectively. *B*, the concentrated red fraction separated from the peroxidase by filtration through calcium phosphate; *C*, the purified red protein, mobility = 2.2 u, with a small spike of lactoperoxidase on the initial falling boundary; *D*, the lactoperoxidase isolated from silica-Celite columns, ratio $A_{412}:A_{280}$ = 0.89; *E*, regenerated lactoperoxidase after acid denaturation, mobility = 2.8 u. Acid denaturation of the lactoperoxidase was accomplished by acidifying to pH 2 with 1 N HCl at room temperature. The red precipitate that formed after addition of 1 N NaOH to pH 5 was centrifuged and washed repeatedly with 0.1 M K_2HPO_4 until the supernatant was free of protein. Regeneration to a soluble red peroxidase was accomplished by suspension in 1 M K_2HPO_4 for approximately 4 months at 3°, during which time the insoluble denatured precipitate gradually dissolved to yield the homogeneous protein solution with one-fifth the original enzymatic activity.

there was a displacement of the colorless protein by the red protein and of the red protein by the green peroxidase. Continued passage of the protein solution in 0.1 M phosphate solution made possible the collection of filtrates containing colorless protein, red protein, and finally the original unfractionated mixture. The use of higher phosphate concentrations (0.2 M) increased the area of adsorbent occupied by a protein band without influencing the order of protein displacement from the column. Finally the protein could be completely eluted from the column with 0.5 to 1.0 M dipotassium acid phosphate solution. As the front of the high salt phase passed through the column, the peroxidase was "peeled off" in a sharp band that increased in protein concentration and color intensity as it moved along the column.

The adsorbent was prepared by an essential modification of Utkin's (12) method. Stoichiometric concentrations of calcium chloride and disodium acid phosphate were combined in solutions made alkaline to phenolphthalein with ammonium hydroxide. The gel was washed free of chloride by decantation over a period of 1 week, filtered, dried overnight at a temperature of 70°, then ground in a mill to a powder fine enough to pass through a standard 90 mesh sieve.

Glass columns, 50 × 4 cm., were packed with 30 to 40 gm. of calcium phosphate contained between layers of glass wool on stainless steel screens. A stainless steel screen on top of the column helped maintain sharp boundaries. Approximately 10 gm. of the adsorbent at one time were tamped until the adsorbent felt firm; excessive pressure was avoided. Some skill is necessary to pack the columns firmly enough to prevent channeling, yet loosely enough to permit the calcium phosphate particles to swell with water without forming an impermeable block. Repeated layers were added to an approximate height of 4 cm. Flow resistance was invariably excessive with longer columns of calcium phosphate. Before use, approximately 50 ml. of water were passed through the column without pressure.

Chromatography of the crude enzyme in solutions of ammonium sulfate gave poor separations on calcium phosphate columns. Control of the ionic composition of the lactoperoxidase by dialysis to a relatively salt-free state and reconstitution to specific phosphate ion concentrations were complicated by the loss of almost half the peroxidase activity during the dialysis procedure. By adjusting the pH of the peroxidase to neutrality with 0.5 M K_2HPO_4, and by dialyzing against 20 volumes of water for 2 hours with continuous stirring of the peroxidase within the membrane, the ammonium sulfate concentration in the crude lactoperoxidase was reduced to about 0.02 M, with 10 per cent loss of enzyme activity. The final solution for chromatography contained 2 per cent protein in 0.1 M K_2HPO_4, pH 9.0. Flow rates of 1 to 3 ml. per minute were attained when this solution was passed over the columns under a pressure of 50 to 80 cm. of mercury.

The chromatographic separation of peroxidase from 100 liters of skim milk is summarized in Table I. In the first run a 0.1 M phosphate solution of the crude enzyme was filtered through four 30 gm. columns under such conditions that the peroxidase-containing filtrate from one column was refiltered through a second column.

Table I

Summary of Isolation Procedure with 100 Liters of Skim Milk

Fraction		Protein	Specific activity determined by: Pyrogallol test	Specific activity determined by: $\frac{A_{412}}{A_{280}}$	Lactoperoxidase yield
		gm.			*mg.*
I. Ammonium sulfate fractionation					
Skim milk		3930	0.094		3030
Whey		715	0.49		2870
(*a*) 2.8 M ppt.		700	0.42		2420
(*b*) 2.0–2.6 M ppt. of (*a*)		533	0.56		2420
(*c*) 1.9–2.3 " " " (*b*)		207	1.09		1850
II. Chromatography					
A. Tricalcium phosphate					
1st run on dialyzed I, *c*					
(*a*) 0.1 M K_2HPO_4 filtrate		154	0.23		290
(*b*) 0.1 " borate wash		17	0.47		657
(*c*) 0.5 " extraction of accidentally broken column*		5.8	4.6		219
(*d*) 0.5 M K_2HPO_4 eluate		4.4	27.0	0.19	974
2nd run on (*d*) diluted to 0.1 M PO_4		*mg.*			
(*e*) 0.1 M K_2HPO_4 colorless filtrate		860	3.3	0.03	23
(*f*) 0.1 " " colored "		1150	3.8	0.03	36
(*g*) 0.1 " borate wash		520	7.3	0.06	31
(*h*) 0.5 " PO_4 eluate, 1st column		910	70.6	0.52	527
(*i*) 0.5 " " " 2nd "		680	42.2	0.31	237
B. Silica-Celite					
1st run ((*h*) + (*i*) diluted to 0.1 M PO_4)					
Combined successive aliquots of 0.5 M K_2HPO_4 effluent	(*a*)	765	95	0.7	595
	(*b*)	444	60	0.6	296
1 M PO_4 effluent	(*c*)	420	41	0.3	140
2nd run ((*b*) diluted to 0.2 M PO_4)					
Combined successive aliquots of	(b_2)	24	115	0.85	17
0.5 M PO_4 effluent	(b_3)	83	95	0.7	65
1 M PO_4 effluent	(b_4)	154	54	0.4	69
3rd run ((b_3) + (*a*) diluted to 0.2 M PO_4)					
0.5 M PO_4 effluent	(a_2)	490	115	0.85	464
4th run ((a_2) + (b_2) diluted to 0.02 M PO_4)					
0.5 M PO_4 effluent	(a_3)	500	122	0.9	500
III. Crystallization of (a_3) with 2.2 M K_2HPO_4					
1st crop (amorphous contaminant)		200		0.89	200
2nd "		250	122	0.90	250

* Adsorbent columns that accidentally became dry during a run invariably developed longitudinal cracks that made them unsuitable for further chromatographic separations. The peroxidase could be recovered by macerating the column in 0.5 M phosphate solution and filtering through a Büchner funnel. Any residual peroxidase was eluted with 1.0 M phosphate.

About three-quarters of the total protein passed through the columns and appeared in the final filtrate. The columns were then washed with 0.1 M sodium tetraborate solution until the borate filtrate was protein-free. This removed any entrained mother liquor and spread the peroxidase band over a larger area of the column, displacing more red protein. Elution of the columns with 0.5 M phosphate gave 500 ml. of effluent containing 1 per cent protein with a 25-fold increase in purity of the peroxidase.

The 0.5 M phosphate elution was continued until the columns were free of protein. Reduction of the phosphate ion concentration by washing with water regenerated the columns, which could be used repeatedly until their flow rates became too slow.

For further purification, the 0.5 M effluent of the first run was diluted with water to 0.1 M salt and 0.2 per cent protein and filtered successively through two columns. The protein impurities displaced from the first column still contained sufficient peroxidase to warrant recovery on the second column. The 0.5 M effluent from the first column had, therefore, a higher purity index than the effluent from the second column. The combined protein from both columns amounted to 1.6 gm. of peroxidase with half maximal purity.

Continued purification with calcium phosphate columns became complicated and involved tedious refractionations of mixtures which became increasingly difficult to resolve. All attempts to elute the red protein selectively failed. Final purification was accomplished more effectively by chromatography on silica-Celite columns. The combined eluates from the calcium phosphate columns were diluted to 0.1 M phosphate and passed through a column composed of a mixture of 2 parts silicic acid[3] to 1 part Celite[4] (13). The permeability of silica-Celite columns imposed less restriction on column height. For reasonably rapid flow rates, pressures of 50 to 80 cm. of mercury were still maintained. When the ratio of 20 mg. of protein to 1 gm. of adsorbent was used, approximately three-quarters the length of a 30 gm. column was saturated with enzyme. Both the lactoperoxidase and the red protein were adsorbed from 0.1 M solutions of dipotassium hydrogen phosphate. Under these conditions, the 0.1 M phosphate filtrate from the columns was free of protein. In contrast to the behavior on calcium phosphate, on silica-Celite the red protein was bound more firmly than lactoperoxidase. As a result, the enzyme was preferentially displaced with 0.5 M phosphate. Spectrophotometric analysis of successive effluent aliquots demonstrated a displacement distribution curve

[3] Merck's reagent grade silicic acid and Johns-Manville Celite analytical filter aid gave reproducible results without any special preparation.

[4] Mention of commercial products does not imply that they are endorsed or recommended by the United States Department of Agriculture over others of a similar nature not mentioned.

similar to that in Fig. 2, protein concentration and purity rising to a sharp maximum that fell off with considerable "tailing." To avoid these "tails," the fraction containing red protein was completely removed from the column with 1 M K_2HPO_4. The red protein was concentrated in this fraction.

The combination of the effluent aliquots into fractions of comparable purity resulted in three main groups, with purity index ranging from 0.7 to 0.3 (Table I, Fraction B, *a*, *b*, *c*).

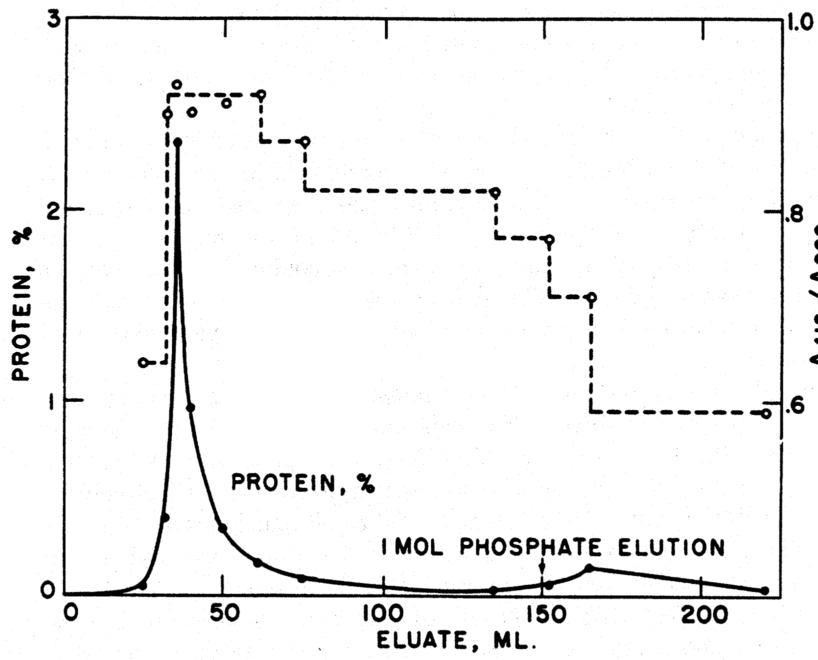

FIG. 2. Elution curve of lactoperoxidase. 266 mg. of lactoperoxidase adsorbed on 10 gm. of silica-Celite from 148 ml. of 0.1 M K_2HPO_4. Ratio, $A_{412}:A_{280}$ of original protein = 0.82. Adsorbed green band displaced with 150 ml. of 0.5 M K_2HPO_4, followed by 1 M K_2HPO_4. ●, plot of the per cent protein eluted against the volume of eluate; ○, ratio $A_{412}:A_{280}$ or purity of the eluted aliquot.

The silica-Celite column was regenerated by continuing the 1 M phosphate elution until the filtrate was free of protein. The excess phosphate was displaced with water, and the column was stored or used immediately.

Readsorption and elution of any one fraction distributed the peroxidase into subfractions of higher and lower purity (Table I, Fraction B, b_2, b_3, b_4). Chromatography of subfraction combinations concentrated the lactoperoxidase through steps of graded purity into the 0.5 M eluate with the absorbancy ratio $A_{412}:A_{280}$ equal to 0.9 (Table I, Fraction B, a_2, a_3). Attempts to increase the peroxidase purity above that of Fraction a_3 by chromatography were unsuccessful.

Crystallization—Purified lactoperoxidase was obtained from the silica-Celite columns as a reddish black solution in 0.5 M dipotassium acid phosphate. The lactoperoxidase was precipitated completely by addition of 4 M dipotassium acid phosphate at room temperature to a final concentration of 2.5 M, and, after addition of approximately 0.5 gm. of Celite (analytical filter aid grade) per 10 ml. of solution, filtered with suction. After elution of the enzyme with 1 M dipotassium acid phosphate, sufficient 4 M dipotassium acid phosphate was added slowly with thorough mixing to a slight turbidity (2.2 M). Approximately 0.2 gm. of Celite per 10 ml. of solution was added and the precipitate was filtered off. The filtrate was stored in a beaker covered with filter paper at 15° for approximately 3 weeks. Crystals that formed were stable in the concentrated phosphate solution for several months at 3° (Fig. 3, *A* and *B*).

Crystallization was accomplished more rapidly with a slightly modified technique. After the first Celite filtration, 4 M phosphate solution was added until a pronounced turbidity formed in the lactoperoxidase. Since the enzyme was more soluble at lower temperatures, most of the precipitate redissolved at 3°. The solution was filtered with Celite at 3° and slowly warmed to room temperature. Crystals formed when the enzyme stood overnight at room temperature (Fig. 3, *E*).

The amorphous precipitates removed by filtration with Celite were recovered by solution in 1 M phosphate and combined with the mother liquor from the crystals for subsequent crystallizations.

Isolation of Red Protein—Electrophoresis of the colored 0.1 M phosphate filtrate from the calcium phosphate columns showed the presence of a multicomponent system containing β-lactoglobulin and at least two other proteins (Fig. 1, *B*). The salmon color of the solution migrated with the component which showed the sharp spike on the falling boundary and had a mobility[5] of 2.4 u in Veronal buffer, pH 8.4, at 0.1 ionic strength. The red fractions obtained from the 0.1 M borate wash or from the 0.5 M eluate of the column cut at the red-green boundary showed similar electrophoretic compositions, but with progressively diminished concentrations of β-lactoglobulin and of the protein with mobility of 4 u. Separation of the red protein from the 4 u protein was accomplished by fractionation with butanol as described by Morton (14), followed by chromatography.

The following procedure made possible the isolation of a relatively pure red protein (Fig. 1, *C*) used to determine the possible relationship between the red protein and lactoperoxidase.

The combined red fractions obtained from the peroxidase isolation were dialyzed to a low salt concentration and adjusted to pH 4.9. The precipitate that formed was filtered off with Celite, and the clear red solution was

[5] The electrophoretic mobility is defined by the unit u, equivalent to sq. cm. per volt per second $\times 10^{-5}$.

shaken with an equal volume of n-butanol at room temperature. After the solution was centrifuged at 20°, the lower red liquid was siphoned off from a voluminous, gelatinous interfacial precipitate. The red fraction was

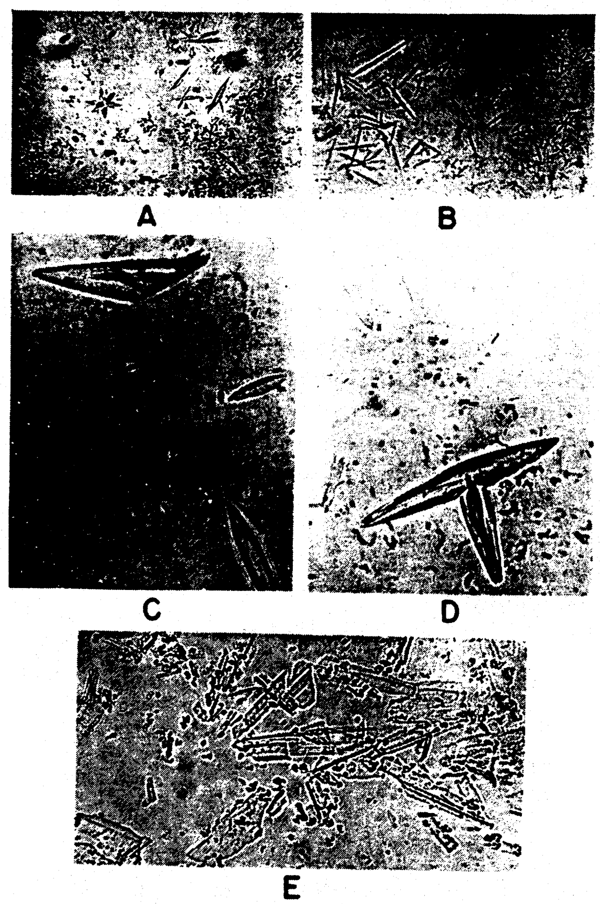

FIG. 3. Crystalline lactoperoxidase in 2.2 M dipotassium acid phosphate. *A* and *B*, first and second crop crystals (120 ×); *C* and *D*, the same crystals (620 ×); *E*, crystals prepared at room temperature (300 ×).

made 0.05 M with dipotassium hydrogen phosphate, and the butanol fractionation was repeated with a quarter volume of the butanol. The lower red solution was filtered clear with Celite and passed over a calcium phosphate column. After the adsorbed protein was washed with 0.1 M borate and 0.1 M dipotassium acid phosphate, the adsorbent was partly dried with air, pushed out of the glass column, and cut into sections at the red-green and red-white boundaries. The red section was eluted with 0.5 M phosphate and the eluate was readsorbed on a second column from 0.1 M phosphate solution. The washed red band was eluted in fractional aliquots based on spectrophotometric analysis with 0.5 M phosphate, yielding the red protein with the electrophoretic composition and mobility indicated in Fig. 1, *C*.

TABLE II

Physicochemical Properties of Crystalline Lactoperoxidase A

Nitrogen, %	15.56
Iron, %	0.069 ± 0.0036
Mol. wt.	82,000

Light absorption

λ	A_s*	λ	A_s*
mμ		*mμ*	
250	0.749	497	0.137
280	1.541	538	0.110
310	0.190	575	0.086
412	1.390	593	0.087
480	0.130	630	0.080

	pH
Isoelectric point	
0.1 μ Veronal buffer	8.0
0.1 " phosphate buffer	6.8
0.05 μ " "	7.6
0.01 " " " (slow component)	8.0
0.1 μ phosphate buffer (fast component)	9.2
Isoionic point	9.6

* A_s = absorbancy index.

II. Physicochemical and Enzymatic Properties

The properties of the isolated lactoperoxidase summarized in Table II are referred to the dry weight of the enzyme dissolved in 0.1 M sodium chloride solution. To remove the phosphate contained in the lactoperoxidase crystals that interfered with the iron determination, the lactoperoxidase crystals were dissolved in water to 0.1 M phosphate solution, pH 8.4, and the enzyme was completely precipitated in amorphous form with an equal volume of acetone at 3°. After centrifugation and decantation, the green precipitate was dissolved in cold 0.1 M sodium chloride and dialyzed against repeated changes of 0.1 M sodium chloride for 3 days. Since the absorbancy index at 280, 310, and 412 mμ did not change significantly after acetone precipitation, no fractionation or denaturation of the enzyme occurred. Evaporation of equivalent aliquots of the 0.1 M salt and peroxidase in salt solutions to dryness and constant weight in a vacuum oven at 80° permitted evaluation of the dry weight of salt-free peroxidase.

Determination of Iron—Attempts to determine the iron in lactoperoxidase with the procedure described by Drabkin (15) were complicated by the formation of a precipitate after adjustment of the pH to 4 with ammonium acetate. Analysis of hemin by the wet ash modification gave reproducible values in close agreement with the theoretical iron content; the procedure[6] was then applied to lactoperoxidase. The reported value of 0.069 ± 0.0036 per cent iron represents the mean value and standard deviation of determinations on seven samples digested separately.

The molecular weight of lactoperoxidase was determined by Dr. M. Halwer of this Laboratory by light-scattering measurements (16) made at 546 mμ on the enzyme in 0.1 M dipotassium hydrogen phosphate at pH 8.4. The solutions, after filtration, were stable on standing overnight in the refrigerator and for at least 3 hours at room temperature. Dilution followed by filtration gave solutions of increased relative turbidity, which was removed by subsequent high speed centrifugation at 40,000 r.p.m. This indicated the formation of aggregates, possibly as a result of denaturation on dilution which could be removed only by centrifugation. The original solution must have contained aggregates, since the turbidity decreased considerably with centrifugation, although there was little accompanying change in concentration or spectral constants.

The first crop of crystals gave a molecular weight of 79,000 with two centrifugations. It is not certain that clarification was adequate with this preparation.

With four centrifugations, the second crop of crystals gave a molecular weight of 84,000 which was unchanged by additional centrifugation. The average molecular weight was 82,000, in close agreement with the value of 81,000 calculated from the iron content of 0.069 per cent, assuming 1 atom of iron per molecule of enzyme.

Light Absorption—The spectral absorption of lactoperoxidase was deter-

[6] 1 or 2 ml. (5 to 10 mg.) of the standard lactoperoxidase preparation in sodium chloride solution were digested with 0.1 ml. of 18 N sulfuric acid by heating on a sand bath to fumes of sulfuric acid. The charred residue was oxidized completely with 3 or 4 drops of 30 per cent hydrogen peroxide and heated again to fumes of sulfuric acid. The completely digested sample was diluted with 10 ml. of water, and the pH was adjusted to 4.2 with 0.6 ml. of 50 per cent ammonium acetate. Color was developed with *o*-phenanthroline after reduction with ascorbic acid according to Drabkin's directions. Optical densities were measured with a Beckman spectrophotometer at 500 mμ and compared with iron standards digested simultaneously with the peroxidase samples.

mined with a Beckman spectrophotometer; cells with absorbing path length of 1 cm. between the boundary layers of solution were used. The photometric scale of the instrument was checked by comparison with the absorbancy of standard potassium chromate solution as reported by Gibson (17). The data are reported in terms of the absorbancy index, $a = ((\log$

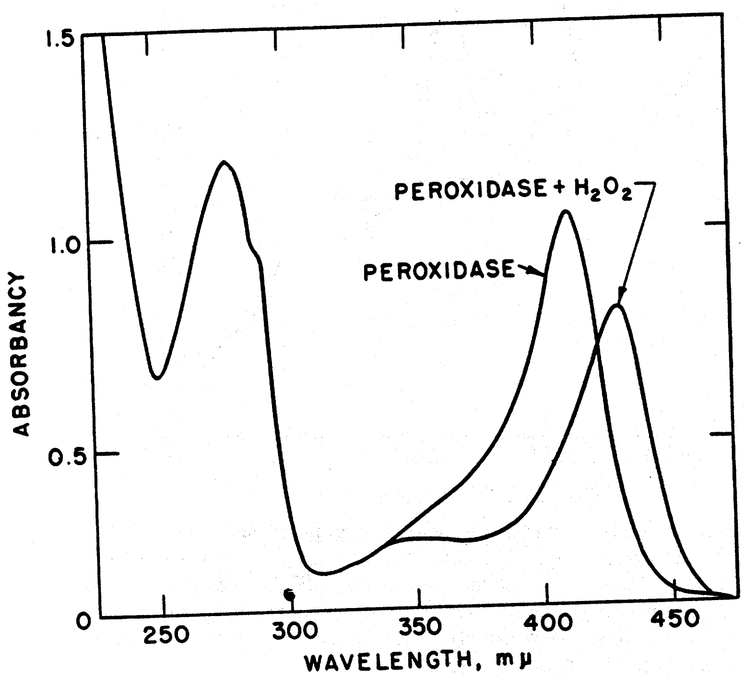

Fig. 4, *A*. Absorption spectrum of lactoperoxidase in 0.1 M phosphate buffer, pH 7.0, obtained with recording spectrophotometer. Concentration of protein, 0.73 gm. per liter; hydrogen peroxide, 8.2×10^{-6} M. Scanning rate, 0.5 mμ per second. Note the wave-length scale change from 325 to 475 mμ.

$I_0/I)/bc)$, where $\log I_0/I$ is the absorbancy (A), b the path length in cm., and c the concentration in gm. per liter (17).

The shape of the absorption spectrum of lactoperoxidase in the ultraviolet and visible regions and the changes produced by hydrogen peroxide are shown in Fig. 4, *A*, and 4, *B*. Use of a Cary recording spectrophotometer made possible the relatively rapid scanning of a spectral region. Although the recording system was too slow to demonstrate a peroxidase I complex with H_2O_2, the more stable peroxidase II complex was easily defined. Comparison of peroxidase II complex with the original enzyme showed no marked variation in the absorption curve other than a shift in the Soret band described by Chance (9).

Qualitatively, the absorption spectrum is identical with Theorell's data for lactoperoxidase, except for the small peak at 290 mμ that may possibly

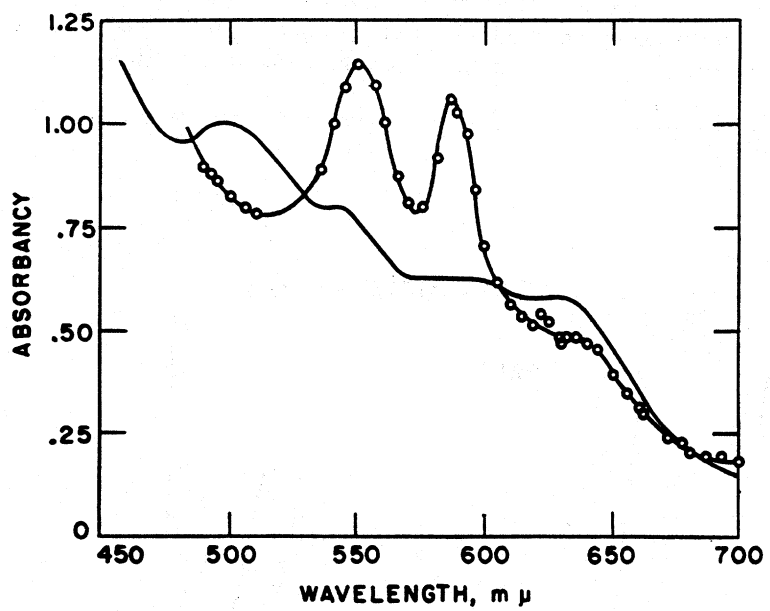

FIG. 4, *B*. Absorption of lactoperoxidase in visible region of spectrum. Same enzyme as in Fig. 4, *A*. Concentration of protein, 7.3 gm. per liter (8.4×10^{-5} M); hydrogen peroxide, 8.2×10^{-5} M, in 0.1 M phosphate buffer, pH 7.0. The points on the upper curve were obtained individually with 100 $\times$ increase in hydrogen peroxide concentration, since at lower concentrations the enzyme hydrogen peroxide complex disappeared too rapidly to be recorded.

TABLE III

Absorbancy Ratios of Enzymes at Indicated Wave-Lengths

Enzyme	Absorbancy ratio		
	280:250 mμ	412:280 mμ	412:310 mμ
Lactoperoxidase (by chromatography)	2.06	0.90	7.3
Theorell and Pedersen's enzyme	1.85	0.77	5.0
Purified red protein	1.78	0.024	0.15

be attributed to the tryptophan content of the enzyme. Quantitatively, the differences are striking and significant, as indicated in Table III.

Lactoperoxidases—Although the results in Table III may be interpreted as the isolation of a purer peroxidase, the possibility remained that the spectral differences reflected structural variations more subtle than mere contamination with foreign protein. Electrophoresis in 0.1 ionic strength acetate buffers (Fig. 5) of a non-crystalline lactoperoxidase preparation, with absorbancy ratio $A_{412}:A_{280}$ of 0.84, showed a distribution of the enzyme into two main components. Spectrophotometric analysis of the isolated fast component on the ascending side and of the slow component on the descending side of the electrophoresis cell showed absorbancy ratio $A_{412}:A_{280}$ to be equal to 0.9 for the fast component and 0.8 for the slow component (Fig. 5, *A*). The electrophoretic separation of two enzymatically active peroxidase components with spectral constants descriptive of Theorell's as well as this investigator's preparations indicated the possible chromatographic isolation of the slow component. Also, inasmuch as The-

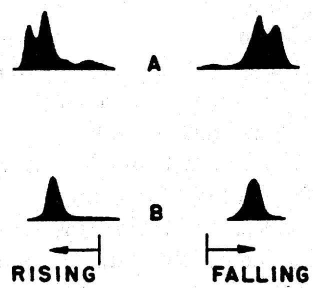

FIG. 5. Electrophoretic patterns of lactoperoxidase preparations in 0.1 ionic strength sodium acetate, acetic acid buffer, after electrophoresis at pH 5.0 for 165 minutes. *A*, winter milk preparation, ratio $A_{412}:A_{280}$ = 0.84. Mobilities of the components on the falling side = 2.84 and 3.94 u, respectively. Spectral ratio $A_{412}:$-A_{280} for the slow component on the falling side = 0.8; for the fast component on the rising side, the ratio = 0.9. *B*, spring milk lactoperoxidase, ratio $A_{412}:A_{280}$ = 0.77; mobility = 2.84 u.

orell's preparation was from spring or early summer milk and the two component enzymes came from winter milk, it appeared reasonable to isolate the slow component lactoperoxidase from spring milk. The fractionation of the spring milk was varied in that the procedure for the isolation of crystalline β-lactoglobulin was applied first to the milk (18). The mother liquor of the first crystallization had an intense green color and contained an appreciable quantity of the milk peroxidase. The enzyme was isolated rapidly by chromatography of the 2.3 M ammonium sulfate precipitate of the mother liquor made alkaline with 0.1 M borate. The lactoperoxidase was different from previous preparations in that repeated chromatography on silica-Celite gave only fractions with a 412:280 mμ absorbancy ratio of 0.77. Electrophoresis of this fraction in acetate buffer at 0.1 ionic strength resolved a single component with a mobility identical with that of the slow component in winter milk lactoperoxidase (Fig. 5, *B*).

For convenience, the lactoperoxidase with ratio $A_{412}:A_{280}$ equal to 0.9

and electrophoretic mobility in 0.1 ionic strength buffer, pH 5, equal to 3.94 u is here called lactoperoxidase A; the enzyme with an absorbancy ratio equal to 0.77 and mobility under the same conditions equal to 2.85 u is called lactoperoxidase B. The possible relationship of the two lactoperoxidase enzymes and the red protein was of interest.

Denaturation Studies—Frequently it was observed that both enzymatic activity and spectrophotometric constants diminished during the course of analytical or preparative procedures. Crystalline lactoperoxidase invariably had lower spectral constants than the constants of the enzyme freshly eluted from the adsorption columns. Lactoperoxidase A, on dialysis to low salt concentration, gradually changed to a protein with spectral constants similar to those of lactoperoxidase B. In an effort to resolve the reactions involved, studies were made on the denaturation of lactoperoxidase by acid, heat, and photooxidation, and the results were correlated with changes in spectral absorption and enzymatic activity.

The changes produced by heat denaturation in the lactoperoxidase molecule are similar to those described by Crammer and Neuberger (19) for a variety of proteins. In general, the changes were more marked in the protein than in the hemin regions of the absorption spectrum. The absorbancy ratios ($A_{412}:A_{280}$, $A_{412}:A_{310}$) decreased with the activity of the enzyme. Concomitantly the protein changed visibly from a green to a red color.

A red protein with one-fifth the original enzymatic activity was also formed by regeneration of acid-denatured and insoluble lactoperoxidase (Fig. 1, *E*). Although the regenerated protein was electrophoretically homogeneous, its mobility in Veronal buffer of 0.1 ionic strength at pH 8.4 was 2.8 u compared to mobilities of 0.2 u for the original lactoperoxidase and 2.3 u for the purified red protein. The red regenerated peroxidase still had a well defined Soret band, with spectral characteristics of $A_{412}:A_{280}$ equal to 0.66 and $A_{412}:A_{310}$ equal to 2.7, as compared to the original values of $A_{412}:A_{280}$ equal to 0.74 and $A_{412}:A_{310}$ equal to 5.0.

Denaturation by a selective photooxidation of the aromatic groups in the peroxidase was made possible by the application of the technique developed by Weil and Buchert (20). The results are shown in Fig. 6. The nature of the spectral changes observed in the ultraviolet is characteristic of the changes found by Weil and Buchert with other proteins. Destruction of the aromatic groups is indicated by the complete disappearance of the 280 mμ band. Although there is a drop in the absorption at 412 mμ, the Soret band is still distinct, as compared with the flat curve found with the red protein in this region. In contrast with the reddish color of both the heat- and the acid-denatured enzymes, the photooxidized protein remained green. The preparation still had about 13 per cent of the original enzyme activity after photooxidation. Results of spectrophotometric and electrophoretic experiments with denatured lactoperoxidase suggest that the release of

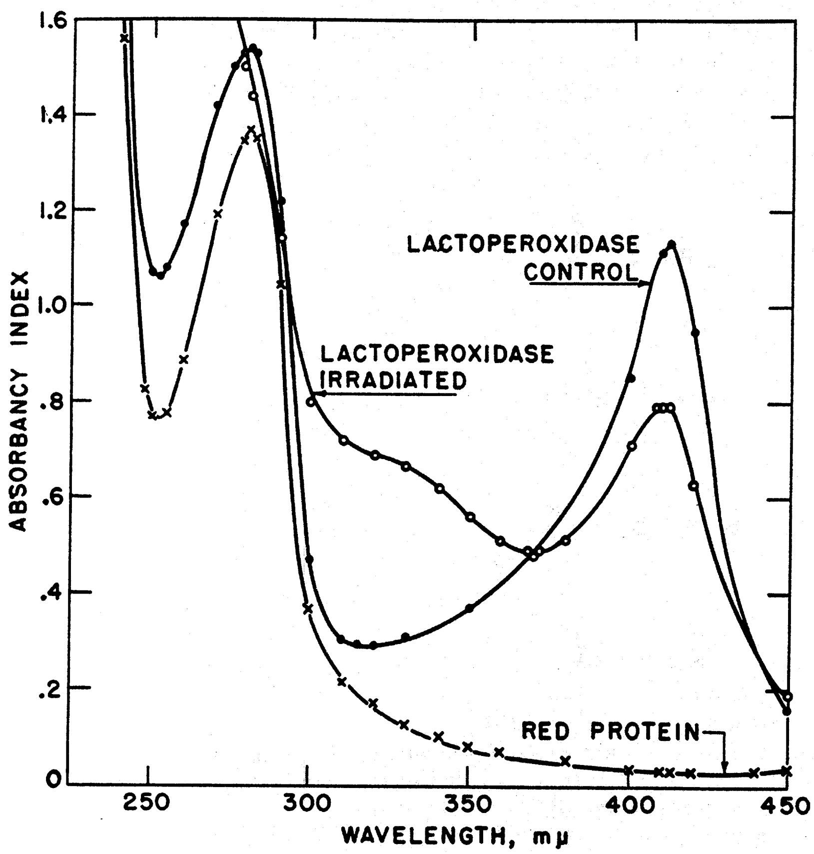

FIG. 6. Absorption spectra of a control lactoperoxidase, the same enzyme after irradiation with visible light in the presence of methylene blue, and a purified red protein. Photooxidation of the enzyme was accomplished in 0.05 M phosphate buffer, pH 8.4. After the uptake of 40 μl. of oxygen per micromole of lactoperoxidase, irradiation with visible light was discontinued. Methylene blue was removed from the enzyme by filtration with charcoal, and the absorption spectrum of the oxidized enzyme was determined and compared with a control lactoperoxidase irradiated without methylene blue.

aromatic group linkages, probably synonymous with the breaking of hydrogen bonds, and the destruction of the hemin group or the coordinated linkages responsible for the Soret band result in the formation of a red lactoperoxidase derivative. There is not sufficient evidence to indicate conclusively that the red protein in milk, found with the lactoperoxidase fraction, is a degradation product of lactoperoxidase. However, the spectral differences between lactoperoxidase A and lactoperoxidase B could be accounted for by a partial oxidation of the aromatic groups in the protein.

Electrophoresis—As in the case of the horse-radish peroxidase I and II isolated by Theorell (21), the results of spectrophotometry and electrophoresis indicated the presence of two lactoperoxidase enzymes, A and B. Electrophoresis of lactoperoxidase A in Veronal buffers of 0.1 ionic strength gave a single component with an average isoelectric point of 8.05 (Fig. 8).

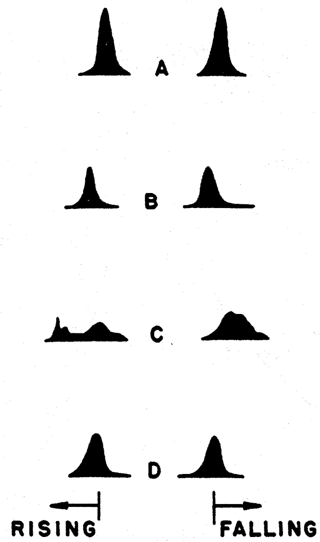

FIG. 7. Electrophoretic patterns of crystalline lactoperoxidase A in phosphate buffers. *A*, 0.1 ionic strength, pH 6.86, mobility = zero; *B*, 0.05 ionic strength, pH 6.95, mobility = 0.66 u; *C*, 0.01 ionic strength, pH 7.0, mobilities = 0.98 and 1.68 u; *D*, spring milk lactoperoxidase in 0.1 ionic strength buffer, pH 6.86, mobility = zero.

The isoelectric point of Theorell's lactoperoxidase was reported as 7.7 in phosphate buffer at 0.1 ionic strength (7).

Electrophoretic studies of lactoperoxidase A, therefore, were made in phosphate buffers that were varied with respect to pH and ionic strength (22). The results of these experiments are illustrated in Fig. 7; the mobilities are summarized graphically in Fig. 8.

In phosphate buffer at 0.1 ionic strength, lactoperoxidase A had an average isoelectric point of 6.9. Decreasing the ionic strength shifted the isoelectric point to a more alkaline pH (Table II). The linear relationship between pH at zero valence and the square root of the ionic strength, reported by Adair and Adair for hemoglobin (23) and by Velick for aldolase (24), held for peroxidase almost to 0.01 ionic strength. The plot of the square root of the ionic strength against the isoelectric point gave a mean extrapolated value of 9.6 for the average isoionic point of lactoperoxidase.

Electrophoresis of the crystalline lactoperoxidase in phosphate buffers at 0.01 ionic strength revealed an electrical heterogeneity that was not manifest at higher ionic strength (Fig. 7, *C*). Varying the pH at 0.01 ionic strength gave isoelectric points at pH 8 for one component and pH 9.2 for the other. Spectrophotometric analysis showed that the ratio $A_{412}:A_{280}$ of the original peroxidase had fallen from 0.89 to 0.82 during the procedures of dialysis and electrophoresis in 0.01 ionic strength buffer. This same

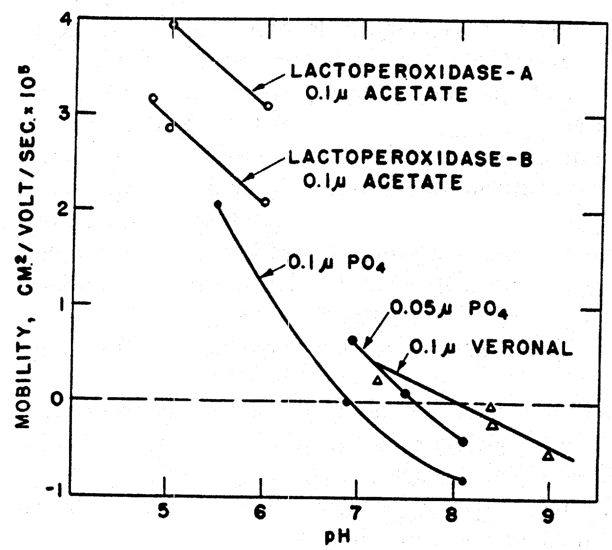

FIG. 8. pH-mobility relationships of lactoperoxidase in acetate, phosphate, and Veronal buffers.

peroxidase, with marked electrical heterogeneity in phosphate buffer of 0.01 ionic strength, showed only a single component when under electrophoresis again in buffer of 0.05 ionic strength. The electrophoresis of spring milk peroxidase (lactoperoxidase B) in phosphate buffer of 0.1 ionic strength at pH 6.86 gave an electrophoretic pattern that could not be distinguished from lactoperoxidase A, in contrast to the results obtained in buffers at higher and lower pH values.

The electrical heterogeneity found with lactoperoxidase reflects a widespread phenomenon in protein chemistry. Crystalline albumin and crystalline β-lactoglobulin represent two of the best examples of "homogeneous" proteins that demonstrate electrical heterogeneity at a particular ionic strength or pH range (25, 26). This splitting of a supposedly pure protein into two or more components is a matter for some concern when investigation of molecular properties, such as molecular weight, or iron content is under consideration. It is conceivable that the small differences in net charge indicated by these components may merely reflect the masking of a dissociable end-group. Until this is demonstrated, the possibility of a gross impurity is real. With lactoperoxidase, the problem is somewhat simplified in that the protein components may be identified by enzymatic activity, spectrophotometrically by chromophoric groups, and also by electrophoretic mobility. The correlated changes in all three characterizations produced by varied physical procedures give some clue at least to the nature of the electrophoretic heterogeneity of lactoperoxidase. Since no specific enzymatic function differentiates lactoperoxidase A from lactoperoxidase B, it appears that the two milk enzymes represent relatively stable energy levels of a catalytically active protein that is degraded into molecular configurations at lower energy levels to a final inactive form.

Enzymatic Activity—Investigation of the pyrogallol reaction for peroxidase activity led to the adoption of the modified Sumner and Gjessing test previously indicated. Although this method was satisfactory for the empirical assay of peroxidase fractions, the high speed of the reaction coupled with an apparent enzyme inactivation by the hydrogen peroxide made it difficult to obtain reliable kinetic data on the pure enzyme with the equipment at our disposal.

Exploring the peroxidative activity of the milk enzyme with other reagents, we observed that, although tyrosine was inactive, hydroxyphenylglycine and dihydroxyphenylalanine reacted with lactoperoxidase and hydrogen peroxide to yield a red oxidation product. Because of the relative stability of dihydroxyphenylalanine (dopa) in water solution and because the color intensity developed at such a rate that absorbancy measurements could be made manually with a Beckman spectrophotometer, dopa was chosen as the reagent for kinetic studies on lactoperoxidase.

Reactions were carried out in 1 cm. square cuvettes holding 3 ml. of solution. The reaction mixture was composed of 0.08 M phosphate buffer, pH 7.0, 16.67×10^{-4} M dopa, 2×10^{-4} M hydrogen peroxide, and a suitable dilution of the enzyme. After final addition of either the dopa or the enzyme, the absorbancy of the oxidized dopa in the reaction mixture was determined every 15 seconds at 475 mμ. With this system as a starting point, the reaction was studied in detail.

The order of addition of reagents apparently determined the order of the reaction. Addition of hydrogen peroxide or enzyme last gave a complicated reaction velocity that followed no simple pattern over the time interval measured. Second order reaction velocity constants held for the 1st minute of the reaction, whereas first order constants decreased linearly with time. When the dopa was added last to the enzyme-hydrogen peroxide complex, a zero order reaction was obtained over the whole 2 minute period. The conditions for a zero order reaction were chosen, therefore, for a detailed study of the reaction.

The relationship between pH and enzyme activity showed a linear increase from no activity at pH 4 to an inflection point at pH 8. At higher pH values, autoxidation of the dopa became increasingly evident, and reliable activity measurements were not possible above pH 9.0. Since the reaction was catalyzed by hemin at alkaline pH, the point of minimal

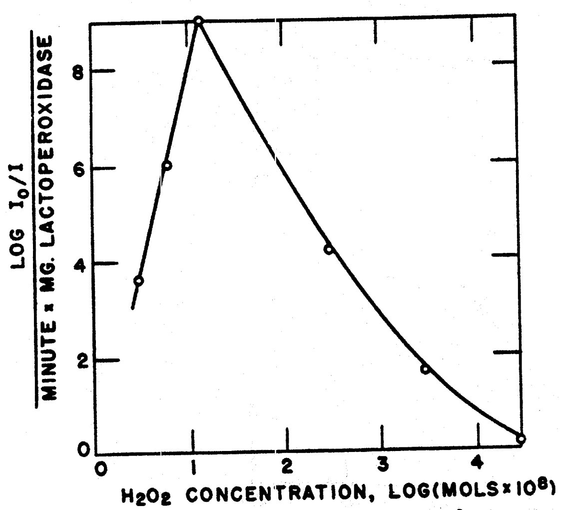

FIG. 9. Effect of hydrogen peroxide concentration on lactoperoxidase-dopa activity. Enzyme concentration constant.

reaction by hydroxyl ion or hemin, namely pH 7.0, was adopted for the enzyme reaction.

As might be expected from the electrophoretic indication of phosphate ion binding to lactoperoxidase, the concentration of phosphate had a pronounced effect on the velocity of the lactoperoxidase reaction with dopa. A sharp increase to maximal activity at 0.08 M phosphate and subsequent decrease to an activity plateau were obtained with the enzyme preparations under the conditions for zero order reaction velocity.

With constant pH, ionic environment, and dopa concentration, the amount of hydrogen peroxide was then varied over a wide range. Since the reaction no longer followed a zero order velocity over the whole concentration range, the activity of the enzyme was arbitrarily taken as the difference between the absorbancy readings at 15 and 60 seconds. As shown in Fig. 9, the enzyme rapidly attained a maximal velocity with increasing amounts of hydrogen peroxide and then decreased to almost complete inhibition.

The inhibition rather than inactivation of the lactoperoxidase with high concentrations of hydrogen peroxide was established by the reactivation of the inhibited enzyme with increased concentrations of dopa. At any one enzyme concentration, the activity of the lactoperoxidase was directly proportional to the ratio of dopa to hydrogen peroxide concentration. This relationship did not reach a maximal value but increased linearly over the experimental range that could be investigated with the limited solubility of the dopa (Fig. 10).

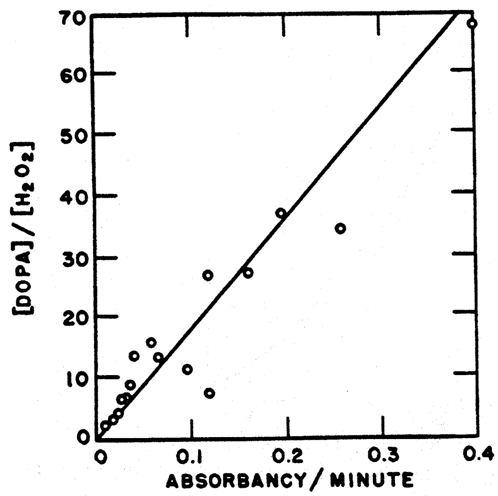

FIG. 10. Relationship between the lactoperoxidase activity and the ratio of acceptor concentration to substrate concentration.

The inhibition of lactoperoxidase activity by hydrogen peroxide and its release with higher concentrations of dopa suggested a type of competitive inhibition for a reactive site on the enzyme. Attempts to demonstrate a dopa-lactoperoxidase complex by spectrophotometry were complicated by the finding that lactoperoxidase had a slow dopa oxidase activity, similar to the action of the enzyme with dioxymaleic acid (27). Observed changes in the spectral band at 290 mμ, occurring with the addition of dopa to the lactoperoxidase, might be attributed, therefore, to the formation of oxidized dopa that did not take place in the control cell without enzyme. Conclusive evidence for the competitive inhibition of the dopa reaction by hydrogen peroxide was obtained from kinetic data. According to the mathematical formulation of Lineweaver and Burk (28), competitive inhibition is indicated by significant changes in the slope of the plot of the reciprocal velocity against reciprocal substrate concentration, without accompanying significant changes in the intercepts of the velocity axis. Fig. 11 represents the double reciprocal plot of the enzyme velocity against concentrations of dopa. Varying the hydrogen peroxide concentration over a 7-fold range produced a family of curves that extrapolated to a common point on or near the origin. This extrapolated point should give the maximal velocity for the enzyme. The anomalous value of infinite velocity must be interpreted to mean that, in the absence of the inhibitory effect of hydrogen peroxide, the velocity of the reaction attains another

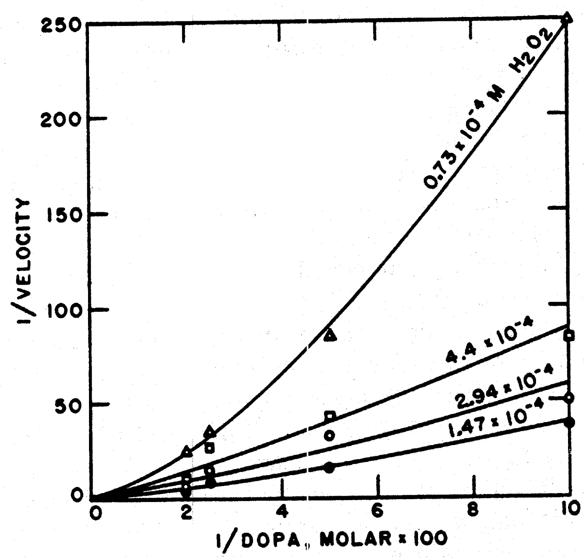

FIG. 11. Double reciprocal plot of the lactoperoxidase activity against the dopa concentrations at various hydrogen peroxide concentrations. 3.475 γ of pure enzyme in each reaction mixture.

order of magnitude that cannot be evaluated with the conventional techniques available to us.

Because of the inhibitory action of hydrogen peroxide, the unit of lactoperoxidase activity with dopa had to be defined on an empirical basis. If the concentration of the enzyme is adjusted to catalyze the utilization of 10 per cent of the hydrogen peroxide per minute, the velocity constant obtained for the zero order reaction may be expressed directly in terms of the turnover number of moles of hydrogen peroxide per mg. or mole of lactoperoxidase per minute. By using a molecular weight of 82,000, the turnover of a pure lactoperoxidase preparation (A_{412}:A_{280} = 0.89) was 1500 moles of H_2O_2 per minute per mole of enzyme. The turnover number of a spring milk lactoperoxide (A_{412}:A_{280} = 0.77) was 1013.

The rate-determining step in the enzymatic activity of lactoperoxidase has been defined by Chance (9) as the reaction of the secondary lactoperoxidase-hydrogen peroxide complex with an acceptor (pyrogallol), since the acceptor controls the transition rate of primary to secondary complex. Although this does not preclude the possibility of a reaction between acceptor and primary complex, with a relatively slow acceptor reaction, this mechanism is valid. The difficulties found with the pyrogallol test for lactoperoxidase were attributed then to the fact that the velocity of the acceptor reaction with complex II approached the speed of the formation of complex I and that the hydrogen peroxide inactivated the enzyme. The modifications of the pyrogallol test suggested by Chance (9) to overcome these difficulties do not attain the effect sought. Primarily, the pyrogallol reaction was troublesome because it was too fast. In this respect, dopa offered a ready solution. The complication of change in reaction velocity with the order of reagent addition and the competitive inhibition imposed by the hydrogen peroxide concentration lead to interesting concepts for the mechanism of peroxidase activity. Conditions for a zero order reaction imply saturation of an enzyme-substrate-dopa complex over the experimental time. This is in keeping with the final addition of dopa to the enzyme-hydrogen peroxide complex for zero order kinetics. The greater velocity obtained when the enzyme or hydrogen peroxide was added last probably indicates that the dopa-enzyme combination was formed before the reaction sites were blocked by hydrogen peroxide. This raises the question of the inhibition of peroxidase activity by the hydrogen peroxide. Although the hemin-catalyzed, dopa-hydrogen peroxide reaction is much slower than the lactoperoxidase reaction, there is no inhibition of the reaction with increased hydrogen peroxide concentrations. The inhibition must, therefore, indirectly result from the iron-protein bond that fixes one of the coordination valences of the iron.

SUMMARY

Lactoperoxidase was isolated in a pure form, and crystallized from 2.2 M dipotassium acid phosphate by salt fractionation at alkaline pH and displacement chromatography on columns of calcium phosphate and silica-Celite.

Chromatography of the peroxidase fraction in phosphate solutions was dependent primarily on the ionic strength of the solution and the specific nature of the adsorbent.

The red protein associated with lactoperoxidase was purified by displacement chromatography.

Light-scattering measurements on the crystalline lactoperoxidase gave a molecular weight of 82,000, in excellent agreement with the value of 0.069 per cent calculated from the iron content.

The absorption spectrum of the isolated lactoperoxidase agreed qualitatively with that reported by Theorell; the absorbancy indices, however, were higher. Particularly significant was the value 0.9 for the absorbancy ratio at wave-lengths 412:280; for Theorell's preparation this value was 0.77.

Electrophoresis of the lactoperoxidase prepared by chromatography coupled with spectrophotometry revealed the presence of two lactoperoxidase enzymes, distinguished by their different mobilities in acetate buffer and their absorbancy ratios at 412 and 280 mμ. Each enzyme was obtained in a form relatively free of the other.

Determination of the isoelectric point of the lactoperoxidase in various buffers revealed the marked binding of phosphate ion by the enzyme, even at alkaline pH. Electrophoresis of single component lactoperoxidase in phosphate buffers at 0.01 ionic strength showed a dissociation of the enzyme into components isoelectric at pH 8.0 and 9.2. The average isoionic point of lactoperoxidase was determined at pH 9.6.

From studies on denaturation by heat, acid, and photooxidation, the possible conversion of lactoperoxidase A to lactoperoxidase B was indicated.

Kinetic studies of the reaction between lactoperoxidase-hydrogen peroxide and dihydroxyphenylalanine revealed a competitive inhibition by the hydrogen peroxide on the peroxidation of the dopa.

From a detailed survey of conditions for the dopa-peroxidase reaction, the activity of lactoperoxidase was defined in terms of the turnover number of hydrogen peroxide. Under conditions for a zero order reaction velocity, this value was 1500 moles per minute per mole of enzyme for lactoperoxidase A.

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